1-1-2016

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Recommended Citation

Moore, D., Hansen, R., & Hand, S. (2016). Liposomes with diverse compositions are protected during desiccation by LEA proteins from Artemia franciscana and trehalose. *Biochimica et Biophysica Acta - Biomembranes, 1858* (1), 104-115. https://doi.org/10.1016/j.bbamem.2015.10.019

Biochimica et Biophysica Acta 1858 (2016) 104–115

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Liposomes with diverse compositions are protected during desiccation by LEA proteins from *Artemia franciscana* and trehalose

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ARTICLE INFO

Article history:
Received 25 June 2015
Received in revised form 2 October 2015
Accepted 26 October 2015
Available online 28 October 2015

Keywords:
Late embryogenesis abundant protein
Trehalose
Liposome
Artemia franciscana
Desiccation
Intrinsically disordered protein

ABSTRACT

Intracellular accumulation of Late Embryogenesis Abundant (LEA) proteins and the disaccharide trehalose is associated with cellular desiccation tolerance in a number of animal species. Two LEA proteins from anhydrobiotic embryos of the brine shrimp *Artemia franciscana* were tested for the ability to protect liposomes of various compositions against desiccation-induced damage in the presence and absence of trehalose. Damage was assessed by carboxyfluorescein leakage after drying and rehydration. Further, using a cytoplasmic-localized (AfrLEA2) and a mitochondrial-targeted (AfrLEA3m) LEA protein allowed us to evaluate whether each may preferentially stabilize membranes of a particular lipid composition based on the protein's subcellular location. Both LEA proteins were able to offset damage during drying of liposomes that mimicked the lipid compositions of the inner mitochondrial membrane (with cardiolipin), outer mitochondrial membrane, and the inner leaflet of the plasma membrane. Thus liposome stabilization by AfrLEA3m or AfrLEA2 was not dependent on lipid composition, provided physiological amounts of bilayer and non-bilayer-forming lipids were present (liposomes with a non-biological composition of 100% phosphatidylcholine were not protected by either protein). Additive protection by LEA proteins plus trehalose was dependent on the lipid composition of the target membrane. Minimal additional damage occurred to liposomes stored at room temperature in the dried state for one week compared to liposomes rehydrated after 24 h. Consistent with the ability to stabilize lipid bilayers, molecular modeling of the secondary structures for AfrLEA2 and AfrLEA3m revealed bands of charged amino acids similar to other amphipathic proteins that interact directly with membranes.

1. Introduction

Organisms adapted to water stress often possess low molecular weight solutes inside their cells that counteract osmotic fluctuation [1,2]. Some of these solutes, such as trehalose, can stabilize biological structures [3–10]. A suite of specialized proteins are also expressed by animals adapted to such conditions. Prominent among these are small stress proteins [11–15] and intrinsically disordered proteins [16,17], the latter group including Late Embryogenesis Abundant (LEA) proteins. In this study, we evaluate the protection of liposomes in the dried state by two LEA proteins from *Artemia franciscana* (AfrLEA3m and AfrLEA2), by the sugar trehalose, and by LEA protein and trehalose in combination. Molecular modeling of AfrLEA3m and AfrLEA2 reveals features consistent with other amphipathic proteins previously demonstrated to interact with lipid bilayers.

LEA proteins are a family of intrinsically disordered proteins that were first discovered in germinating cottonseeds in 1981 [18]. Since that time they have been found in seeds and desiccation tolerant tissues of many plant species [19]. In recent years, LEA proteins have been found to accumulate during dehydration tolerant stages of development in a number of non-plant organisms. These include prokaryotes [20], rotifers [21], nematodes [22], larvae of an insect (*Polypedilum vanderplanki*, the African chironomid [23]), and embryos of the brine shrimp (*Artemia franciscana* [24]). Many LEA proteins are unstructured in solution and adopt a secondary structure as water is removed. Due to a biased amino acid composition, LEA proteins are extremely hydrophilic. LEA proteins are divided into 6 groups based on possession of repeated sequence motifs. The majority of LEA proteins found in animals belong to Group 3 [19,25,26]. *In vitro* experiments indicate that LEA proteins may perform several functions in desiccation-tolerant cells. During drying, LEA proteins protect target enzymes [27,28] and prevent protein aggregation [29,30]. Several LEA proteins form amphipathic alpha-helices are capable of interacting with lipid bilayers [29,31–33], although membrane stabilization during water stress has only been experimentally demonstrated for three LEA proteins from plants: PsLEAm (drying), COR15a and COR15b (freezing; also known as LEA23 and LEA24) [32,34–36]. Additionally, LEA proteins may form

Abbreviations: CF, 5(6)-carboxyfluorescein; CL, cardiolipin; ILPM, inner leaflet of plasma membrane; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PC, phoshphatidylcholine; PE, phosphatidylethanolamine, and PI, phosphatidylinositol; PS, phosphatidylserine; TES, 2-[[1,3-dihydroxy-2-(hydroxymethyl)-2-propanyl]amino]ethanesulfonic acid; T_m, phase transition temperature

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http://dx.doi.org/10.1016/j.bbamem.2015.10.019
0005-2736/

structural networks [37] and strengthen glasses formed by protective sugars [38].

Targeting of LEA proteins to different compartments within the cell explains some of the multiplicity of LEA proteins within a given species [26]. Subcellular localization emphasizes the necessity of protecting organelles from water stress-induced damage. To date, mitochondria-targeted LEA proteins have been identified definitively in two species of plants (*Pisum sativum*, the pea plant [27] and *Arabidopsis thaliana* [39]) and one animal, the brine shrimp *Artemia franciscana*. The identification and predicted mitochondrial localization of AfrLEA3m from *A. franciscana* was first reported by Menze et al. [40]. Experimental evidence now supports a mitochondrial location for AfrLEA3m, based on Western blots of isolated mitochondria and immunohistochemistry of embryos [41,42]. Interestingly, *A. franciscana* mitochondria contain four isoforms of AfrLEA3m and at least two Group 1 LEA proteins [42–46]. Functionally, AfrLEA3m protects target enzymes against desiccation-induced damage [28], and when transfected into human HepG2 cells, preserves membrane integrity and viability after acute desiccation and rehydration [47].

Currently, no cytoplasmic-localized LEA protein has been reported to stabilize lipid bilayers *in vitro*. AfrLEA2 is located in the cytoplasm of *A. franciscana* embryos [24,41], and shares similar abilities with ArfLEA3m to protect target enzymes and HepG2 cells during drying [28,47]. It has been suggested that a given LEA protein may preferentially stabilize membranes of a particular lipid composition based on the protein's subcellular location [31,32,48]. Using both cytoplasmic-localized and mitochondrial-targeted LEA proteins to study protection of liposomes during drying should allow a direct test of this concept. For example, one prediction would be that AfrLEA3m will stabilize liposomes mimicking the inner mitochondrial membrane to a greater degree than AfrLEA2.

Numerous studies have demonstrated the ability of trehalose to stabilize biological structures subjected to desiccation and freezing [3,7–9]. Trehalose is even more effective at stabilizing lipid bilayers during freezing than during drying. The efficacy of trehalose during severe dehydration is partially attributed to its ability to form sugar glasses [8,49] and to replace the hydration water normally associated with phospholipid head groups of lipid bilayers and with proteins, i.e., the 'water replacement hypothesis' [50,51]. Damage to lipid bilayers during drying is primarily attributed to two stresses: fusion and phase transitions [52]. Trehalose has been documented to protect both liposomes and native membranes [8,9,47,52–54]. Trehalose prevents membrane fusion of dried liposomes, presumably due to its ability to foster glass formation [8,49,55]. The phase transition temperature (T_m) of lipid bilayers increases dramatically during desiccation, such that the liquid-crystalline state can convert to a gel state at room temperature. Bilayers become leaky during phase transitions [56]. In order to depress the T_m and prevent damaging phase transitions during drying and rehydration, and the sugar must be present on both sides of the bilayer for optimal protection [57]. One proposed mechanism for the strong depression of T_m by trehalose is the replacement of water bound to phospholipid head groups as discussed above.

In the present study, liposomes with compositions simulating the inner mitochondrial membrane, the outer mitochondrial membrane, and inner leaflet of the plasma membrane were air dried with and without AfrLEA2, AfrLEA3m and trehalose. Additionally, each LEA protein was tested in combination with trehalose to determine whether liposome protection (as assessed with a carboxyfluorescein leakage assay) would be enhanced beyond that afforded by LEA protein alone; additivity and in some cases synergistic effects have demonstrated in drying studies with target enzymes [28,30]. Both LEA proteins clearly stabilized all three types of liposomes significantly better than a control protein (lysozyme). However, there were no clear differences in the degree of protection afforded by either LEA protein that were correlated with specific liposome compositions.

2. Materials and methods

2.1. Biochemicals

Lipids and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). All lipids were purified from the specified tissue sources in order to mimic the fatty acid distribution found in eukaryotic cells. Phoshphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were purified from bovine liver (Avanti Polar Lipids; product numbers 840055, 840026, 840042). Because these phospholipids were naturally derived, they contained a mixture of fatty acids. However, the predominant phospholipid species in each sample above was confirmed by the supplier to be 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine, and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol. Phosphatidylserine (PS; predominantly 1-stearoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine) was purified from bovine brain tissue (Avanti Polar Lipids; product number 840032) and cardiolipin (CL; predominantly 1′,3′-bis[1,2-dilinoleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol) from bovine heart (Avanti Polar Lipids; product number 840012). Cholesterol was isolated from ovine wool (Avanti Polar Lipids; product number 700000P). 5(6)-Carboxyfluorescein (CF) (product number 21877) was obtained from Sigma-Aldrich (St. Louis, MO). Trehalose (product number T-104-4) was purchased from Pfanstiehl (Waukegan, IL)..

2.2. Liposome preparation

Lipids were combined with mass ratios simulating the inner mitochondrial membrane, outer mitochondrial membranes, and inner leaflet of the plasma membrane of mammalian cells. The lipid compositions of mammalian membranes are well-defined [58,59], and these membranes are known to be sensitive to water stress-induced damage [9,56]. Consequently the lipid contents of these membranes were used as a first approximation to prepare representative liposomes (Table 1). For comparison to a non-physiological bilayer, liposomes composed entirely of phosphadidylcholine were also prepared. Lipid mixtures were dried under a stream of nitrogen, and then stored under vacuum overnight to remove residual solvent. For a typical batch, 20 mg of total lipids were used. Lipids were rehydrated in 1 ml of 100 mM CF, 10 mM TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)-2-propanyl]amino]ethanesulfonic acid), 0.1 mM EDTA (pH 7.4) for at least 1 h with intermittent vortexing at 70 °C, a temperature sufficiently high to ensure all phospholipids were in the liquid crystalline phase. Then large unilamellar vesicles (LUVs) were prepared with a hand held mini extruder (Avanti Polar Lipids, Alabaster, AL). Lipid preparations were passed through two stacked polycarbonate membranes with 1 μm pore size prior to passage through two stacked membranes with 0.1 μm pore size at 70 °C. Liposomes were extruded through the 0.1 μm membranes at least 10 times to ensure uniform size distribution. After extrusion, liposomes

Table 1
Composition of liposomes used to mimic mammalian membranes. Mixtures are expressed as percent (w/w).

Mammalian membrane	Composition of liposomes	Reference
Inner leaflet of the plasma membrane	50% PC 30% PE 10% PS 10% Cho	[57]
Inner mitochondrial membrane	50% PC 30% PE 20% CL	[58] [32]
Outer mitochondrial membrane	55% PC 30% PE 15% PI	[58]

Cho — cholesterol; CL — cardiolipin; PC — phosphatidylcholine; PE — phosphatidylethanolamine; PI — phosphatidylinositol; PS — phosphatidylserine.

were eluted through a Sephadex G-25 column (NAP-5, GE Healthcare, Buckinghamshire, UK) that was equilibrated in TEN buffer (50 mM NaCl, 10 mM TES, 0.1 mM EDTA, pH 7.4) to remove CF not entrapped by the liposomes. Liposomes were mixed with 250 mM trehalose (19:1 sugar:lipid mass ratio) and/or LEA proteins to yield various protein:lipid mass ratios between 0.1 and 0.4. For some experiments (i.e., Fig. 2D), trehalose was included during the preparation of LUVs, so that the sugar also was present inside of the liposomes. The final concentration of liposomes was approximately 5 mg total lipid/ml.

In order to compare the impact of LEA proteins to a negative control (i.e., a protein predicted to be non-stabilizing), liposomes were also dried with lysozyme at identical protein:lipid mass ratios. Lysozyme was chosen because it retains its native structure when dried and does not interact with membranes [60,61]. Finally, it is appropriate to note that desiccation-tolerant embryos of brine shrimp contain large concentrations of trehalose [62–64], and consequently it is relevant to include this solute in the present studies of liposome protection by LEA proteins that originate from the same species.

2.3. Air drying and CF leakage measurements

For CF leakage assays, 2 μl droplets of liposome mixtures were placed in the wells of opaque 96-well plates (OptiPlate 96-F, PerkinElmer, Waltham, MA). Droplets were air dried in the dark overnight or for one week at room temperature in an airtight desiccation cabinet (Fisher Scientific, Hampton, NH) containing Drierite (calcium sulfate) [32]. Droplets were rehydrated by the addition of 300 μl of TEN buffer at room temperature and aspirated by pipet to achieve a uniform suspension. Fluorescence of CF is strongly quenched at the high concentration of 100 mM contained inside of the liposomes, but increases when CF is released into the medium [65]. After measuring CF fluorescence of rehydrated liposomes (F_0), Triton X-100 was added to a final concentration 1% in order to fully lyse the liposomes (F_T). The percent CF leakage was calculated as $(F_0/F_T) \times 100$. Measurements were made with a Victor 3 Multilabel Counter (PerkinElmer, Waltham, MA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. In the presence of 1% Triton X-100, significant quenching of CF fluorescence was observed. Therefore, F_T measurements were corrected using standard curves of CF fluorescence versus known concentrations of the dye in the presence and absence of detergent. While this correction was essential, it sometimes led to control values (no protectant) slightly above 100% CF leakage.

2.4. LEA protein purification

Recombinant AfrLEA3m and AfrLEA2 were purified as described by Boswell et al. [42]. Briefly, an ACTA Prime Plus FPLC (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used for Ni^{2+} affinity chromatography with a HisTrap FF crude column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with a mobile phase consisting of 20 mM imidazole, 500 mM NaCl and 20 mM sodium phosphate, pH 7.5. Bound protein was eluted with 500 mM imidazole prepared in the mobile phase buffer. The sample was then dialyzed overnight against 10 mM NaCl containing 20 mM triethanolamine, pH 7.0 and applied to a HiTrap Q FF column (GE Healthcare Bio-Sciences AB) equilibrated with this same buffer. Bound protein was eluted with a NaCl gradient from 10 mM to 1 M.

2.5. Modeling of AfrLEA3m and AfrLEA2

Primary amino acid sequences of AfrLEA3m and AfrLEA2 were determined from our existing cDNA library for *A. franciscana* as published previously (24, 40; GenBank accession no. ACA47268.1 for AfrLEA2, ACM16586.1 for AfrLEA3m). Sequences were modeled as α-helices using UCSF Chimera version 1.10, which was obtained at cgl.ucsf.edu/chimera. Images of the resulting protein model were generated with DeepView version 4.1 (formerly Swiss-PDB Viewer), which was downloaded from spdbv.vital-it.ch.

2.6. Statistics

Two-way ANOVA paired with a Tukey post-hoc test were used to compare protection of each type of liposome by AfrLEA3m, AfrLEA2 or lysozyme across the range of protein:lipid mass ratios tested (Prism 6; GraphPad Software, La Jolla, CA). A Bonferroni multiple comparisons test of sample means was used to identify statistical differences between trehalose and protein combinations compared to each component alone (Prism 6). Multiple t-tests combined with a Holm–Sidak post hoc test were used to compare CF leakage from liposomes after short-term versus long-term drying (Prism 6). Significance level was set at $p \leq 0.05$.

3. Results

3.1. Short-term drying of liposomes with LEA proteins

Stability of liposomes during drying and rehydration was assessed by measuring leakage of entrapped carboxyfluorescein (CF) upon rehydration. Leakage from liposomes simulating the inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM) or inner leaflet of the plasma membrane (ILPM) was reduced in a concentration dependent manner by both AfrLEA2 and AfrLEA3m (Fig. 1). In order to compare multiple liposome mixtures, leakage was normalized to that of control liposomes, which were dried without any protectant. When liposomes with realistic biological compositions were dried overnight with AfrLEA2 or AfrLEA3m at a 2:5 (protein:lipid) mass ratio, liposomes retained 38–48% more of entrapped CF than liposomes dried without any protectant (Fig. 1A and B). By comparison, lysozyme (a control protein known for its lack of interaction with lipids, see Materials and Methods), did not exhibit concentration-dependent protection of any liposome tested; liposomes retained only ~13% of entrapped dye at all protein:lipid ratios tested. When dried with LEA proteins, retention of entrapped dye by liposomes mimicking biological membranes was significantly greater at all protein:lipid mass ratios compared to liposomes dried with lysozyme (2-way ANOVA, $p \leq 0.05$, $n = 6$). Non-biological membranes composed of 100% phosphatidylcholine (PC) leaked 76–85% of entrapped CF when dried with LEA proteins at the highest protein concentrations tested. CF leakage in the presence of LEA proteins was not significantly less than leakage from PC liposomes dried with the same amount of lysozyme except at the highest concentration of AfrLEA2.

3.2. Short-term drying of IMM-like liposomes with trehalose and LEA proteins

When trehalose was present only outside of liposomes that simulated the IMM, the sugar provided little protection (CF leakage, 95.7 ± 1.6%; mean ± SD, n = 3–6) (Fig. 2). These results are similar to other liposome drying studies where trehalose was confined to the outside of liposomes [55,66]. At the highest concentration tested with AfrLEA2 alone, CF leakage was depressed to 64.2 ± 0.4% (mean ± SD, n = 3–6) for IMM liposomes dried and rehydrated (Fig. 2A). At the same concentration of AfrLEA2 but with the addition of 250 mM trehalose outside, CF leakage decreased to 56.1 ± 0.8%. The amount of protection afforded by the combination of AfrLEA2 and trehalose was significantly greater than that with either component alone at the highest and lowest protein:lipid mass ratios tested (2-way ANOVA, Bonferroni multiple comparisons test, $p \leq 0.05$, $n = 6$). AfrLEA3m alone decreased CF leakage from IMM liposomes to 65.2 ± 2.1% (mean ± SD, n = 3–6) at the highest protein:lipid mass ratios tested (Fig. 2B). When both trehalose and AfrLEA3m were present, CF leakage was further decreased to 56.6 ± 6.1% (mean ± SD, n = 3–6) at the same protein:lipid mass ratio

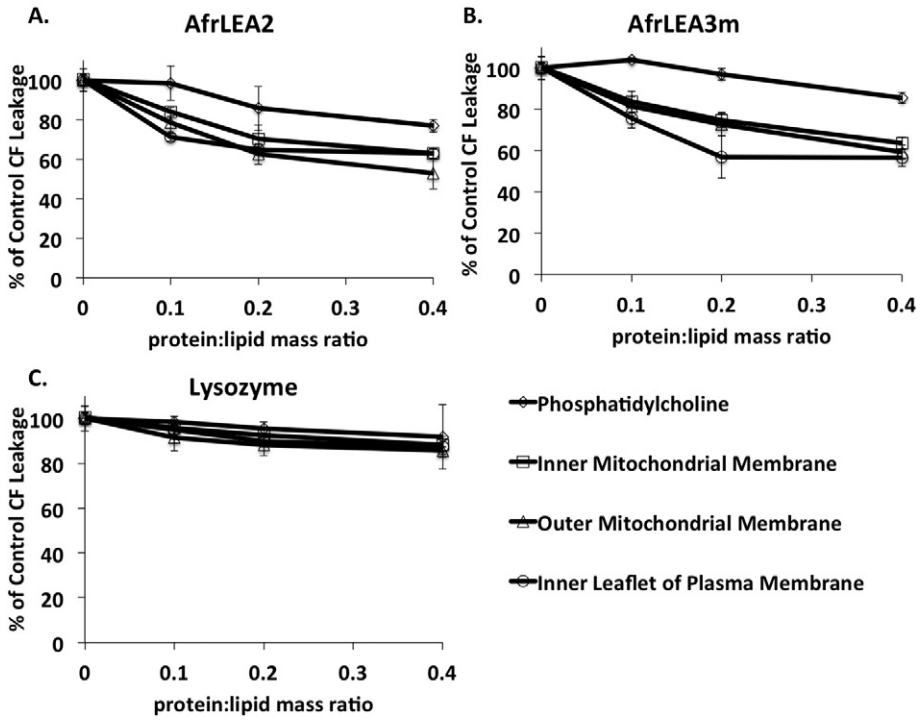

Fig. 1. Dye leakage from liposomes dried overnight and rehydrated in the presence of LEA proteins and lysozyme. The relative stability of the membranes was determined by measuring the loss of entrapped fluorescent dye carboxyfluorescein (CF), expressed as a percentage of complete dye release quantified by detergent solubilization. Experimental treatments were then normalized to control treatments (no protectant), which was necessary due to inherent differences in control leakage across compositional types. Lipid compositions simulated those of the inner mitochondrial membrane, the outer mitochondrial membrane, the inner leaflet of the plasma membrane, or a non-biological composition of 100% phosphatidylcholine. Liposomes were dried with the indicated protein:lipid mass ratios of (A) AfrLEA2, (B) AfrLEA3m, (C) lysozome. Data represent the mean ± SD of n = 6 samples; where error bars are absent, the SD was less than the size of the symbol. Statistical analysis was omitted from figures for clarity. At all protein:lipid mass ratios, stabilization by LEA proteins was significantly greater than that afforded by lysozyme for all membranes except 100% PC liposomes (2-way ANOVA, $p \leq 0.05$, n = 6). In the case of 100% PC liposomes, improved stabilization was only seen at the highest concentration of AfrLEA2.

(Fig. 2B). CF leakage was significantly lower at all protein:lipid mass ratios tested (2-way ANOVA, $p \leq 0.05$, n = 6) when trehalose and AfrLEA3m were both present compared to either component alone; thus protection by these factors was additive. The presence of lysozyme also enhanced the ability of external trehalose to stabilize IMM-like liposomes (Fig. 2C), but the overall impact was small; CF leakage was only depressed to 76.9 ± 0.6% (mean ± SD, n = 3–6) at the highest lysozyme:lipid ratio tested in the presence of 250 mM trehalose outside, which was far less protection than that afforded by LEA proteins alone or in combination with trehalose..

In order to quantify maximal stabilization by trehalose, IMM-like liposomes were also prepared with 250 mM trehalose present inside of the liposomes as well as outside (Fig. 2D). Not surprisingly, when dried with both internal and external trehalose, CF leakage was diminished to 38.9 ± 0.6%, which was a 56% decrease in leakage compared to liposomes dried with external trehalose only (Fig. 2D). Furthermore, overall CF leakage was significantly lower than that seen with any combination of LEA protein and trehalose when these constituents are restricted to the outside of liposomes. No further improvement in CF retention was observed when liposomes containing 250 mM trehalose inside were dried with both trehalose and LEA proteins present outside in the medium (data not shown).

3.3. Short-term drying of ILPM-like liposomes with LEA proteins and trehalose

When dried in the presence of external trehalose, ILPM-like liposomes leaked 93.3 ± 7.9% (mean ± SD, n = 6) of entrapped CF (Fig. 3). The greatest protection by AfrLEA2 alone reduced CF leakage to 65.2 ± 4.2% (mean ± SD, n = 6) (Fig. 3A). When AfrLEA2 and extrernal trehalose were used in conjunction, protection was significantly greater than with either component alone at the lower concentrations of AfrLEA2 tested but not at the highest (2-way ANOVA, Bonferroni multiple comparisons test, $p \leq 0.05$, n = 6). AfrLEA3m depressed CF leakage from ILPM-like liposomes to 58.6 ± 4.3% (mean ± SD, n = 6) (Fig. 3B). Unexpectedly, the addition of external trehalose did not significantly increase protection by AfrLEA3m at any of the protein:lipid mass ratios tested. Once again, protection by lysozyme and external trehalose was found to be additive. However, maximal protection by lysozyme and trehalose (CF leakage, 70.2 ± 4.9%; mean ± SD, n = 6) was statistically less than that of liposomes dried with AfrLEA2 in conjunction with external trehalose (2-way ANOVA, Bonferroni multiple comparisons test, $p \leq 0.05$, n = 6).

3.4. Short-term drying of OMM-like liposomes with trehalose and LEA proteins

Liposomes that simulated the OMM lost 81.0 ± 6.3% (mean ± SD, n = 6) of entrapped CF when dried in the presence of 250 mM trehalose outside (Fig. 4). CF leakage was depressed to 53.2 ± 7.8% (mean ± SD, n = 6) when OMM-like liposomes were dried with AfrLEA2 (Fig. 4A). The combination of trehalose and AfrLEA2 did not significantly improve stability of OMM-like liposomes. Stabilization of OMM model liposomes by AfrLEA3m was similar to that of AfrLEA2; maximal protection resulted in 59.8 ± 3.4% CF leakage (mean ± SD, n = 6) (Fig. 4B). Once again, no additivity occurred during drying when 250 mM trehalose was included in the medium with AfrLEA3m. When OMM-like liposomes were dried with lysozyme and trehalose, CF leakage was not statistically different than with 250 mM trehalose alone, but leakage was significantly reduced compared to lysozyme alone (2-way ANOVA, Bonferroni multiple comparisons test, $p < 0.05$, n = 6) (Fig. 4C). Stability of OMM-like liposomes dried with a combination of lysozyme and 250 mM trehalose was less than that for AfrLEA3, except at the highest lysozyme concentration (2-way ANOVA, Bonferroni multiple comparisons test,

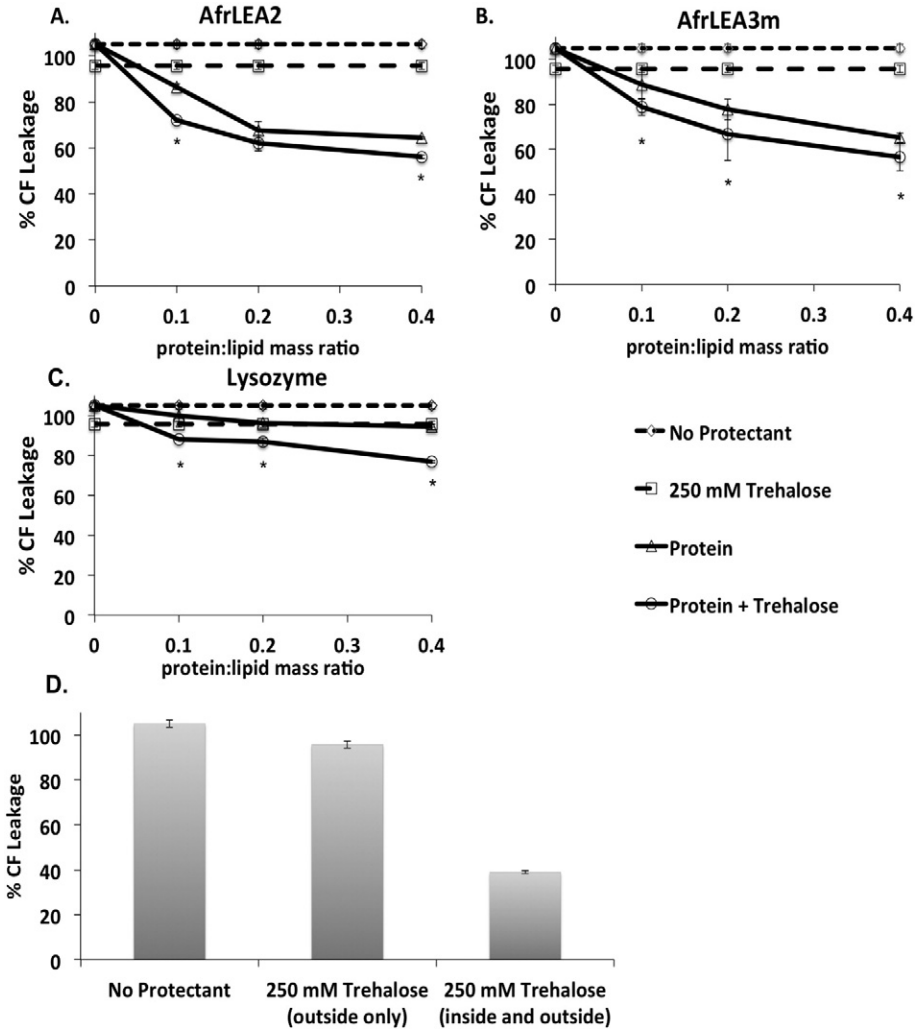

Fig. 2. Dye leakage from liposomes simulating the inner mitochondrial membrane dried overnight and rehydrated in the presence of trehalose, LEA proteins and lysozyme. The relative stability of the membranes was determined by measuring the loss of entrapped fluorescent dye carboxyfluorescein (CF). CF leakage was expressed as a percentage of complete dye release, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were added to drying media at the indicated protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme as a control protein. Data represent mean ± SD of n = 3–6 samples; where error bars are absent, the SD was less than the size of the symbol. Asterisks indicate significantly greater protection by trehalose and protein combined than either component alone (2-way ANOVA, Bonferroni multiple comparisons test, $p \le 0.05$, n = 3–6). (D) IMM-like liposomes were dried with 250 mM trehalose present on both sides of the lipid bilayer for comparison to liposomes dried without protectant and with trehalose present only outside of the liposomes. Data represent the mean ± SD of n = 6 samples.

$p \le 0.05$, n = 6). Maximal protection by AfrLEA2 at all protein concentrations tested was greater than that of lysozyme and external trehalose (2-way ANOVA, Bonferroni multiple comparisons test, $p < 0.05$, n = 6).

3.5. Short-term drying of 100% PC liposomes with trehalose and LEA proteins

Liposomes composed of 100% PC were prepared in order to assess whether LEA proteins would protect a bilayer with a non-biological composition. Loss of CF from PC liposomes dried with LEA proteins was not less than leakage from PC liposomes dried without any additive except at the highest protein:lipid mass ratio tested for both ArfLEA2 and AfrLEA3 (2-way ANOVA, Bonferroni multiple comparisons test, $p \le 0.05$, n = 6). PC liposomes dried overnight with AfrLEA2 lost 69.0 ± 2.3% (mean ± SD, n = 6) of entrapped CF and 76.6 ± 2.4% with AfrLEA3m (Fig. 5A and B). Leakage from PC liposomes dried with lysozyme was equivalent to that for LEA proteins except at the highest protein:lipid mass ratio tested, where leakage with lysozyme was greater than that with AfrLEA2 (2-way ANOVA, Bonferroni multiple comparisons test, $p \le 0.05$, n = 6). There was no significant difference in CF leakage from PC liposomes when trehalose was used in conjunction with either LEA protein compared to protection by the proteins alone.

3.6. Long-term drying of liposomes with trehalose and LEA proteins

The stability of dried liposomes after 7 days of storage at room temperature and 0% relative humidity was also examined (Fig. 6). Protection by combinations of trehalose, AfrLEA2, and AfrLEA3m was evaluated for all four liposome compositions; CF leakage from liposomes dried with lysozyme or without any protectants was also measured. With only four exceptions, significant increases in CF leakage were not observed after 7 days of drying compared to liposomes dried overnight (multiple t-test, Holm–Sidak post hoc test, $p \le 0.05$). When there were significant increases with extended drying, the magnitude of the effects was very modest. Most cases for which CF leakage increased were observed for OMM-like liposomes, i.e., those dried with lysozyme, AfrLEA2, or AfrLEA3m at a 0.4 protein:lipid mass ratio (multiple t-test, Holm–Sidak post hoc test, $p \le 0.05$). These increases in CF leakage from OMM-like liposomes did not occur when 250 mM trehalose was

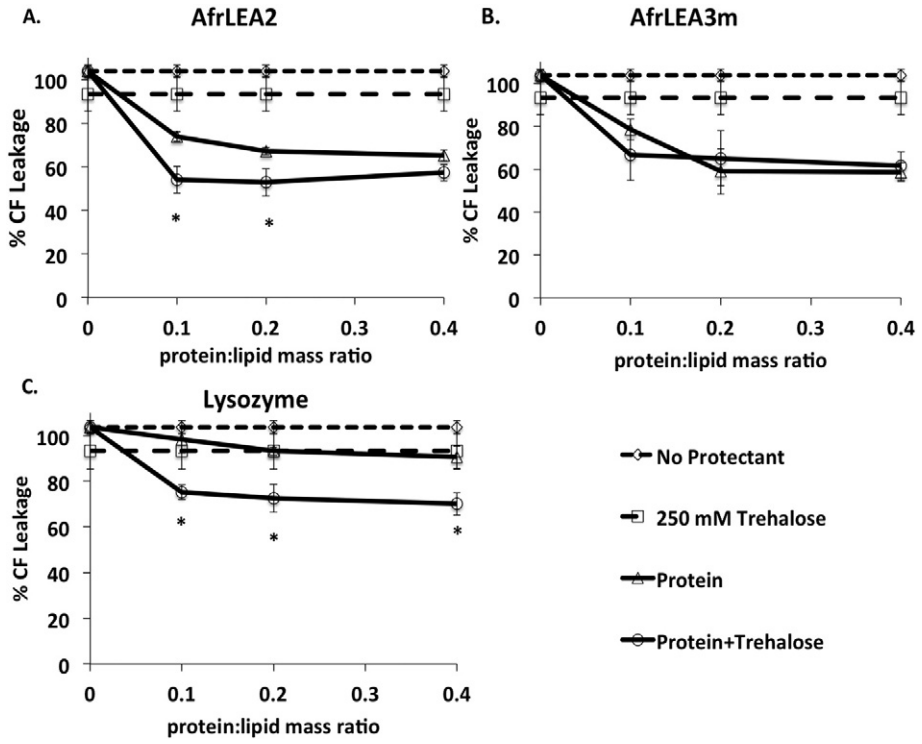

Fig. 3. Dye leakage from liposomes simulating the inner leaflet of the plasma membrane dried overnight and rehydrated in the presence of trehalose, LEA proteins, and lysozyme. The relative stability of liposomes was determined by measuring the loss of entrapped carboxyfluorescein (CF). CF leakage was expressed as a percent of total CF loss, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were added to drying media at the indicated protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme, a non-stabilizing protein, for comparison. Data represent mean ± SD of n = 3–6 samples; where error bars are absent, the SD was less than the size of the symbol. Asterisks indicate significantly greater protection by trehalose and protein combined than either component alone (2-way ANOVA, Bonferroni multiple comparisons test, $p \le 0.05$, n = 6).

also present in the external medium (multiple t-test, Holm–Sidak post hoc test, $p \le 0.05$). The fourth exception to the general lack of increased CF leakage after extended drying occurred with 100% PC liposomes dried with AfrLEA2 and external trehalose (multiple t-test, Holm–Sidak post hoc test, $p \le 0.05$).

3.7. Molecular modeling of AfrLEA3m and AfrLEA2

Drying of AfrLEA3m and AfrLEA2 promotes formation of substantial *α*-helical structure [28]. Therefore, models of AfrLEA3m and AfrLEA2 were generated as helical rods (Figs. 7 and 8). The distribution of acidic and basic residues revealed a distinctive organization associated with amphipathic proteins that interact with lipid bilayers [67–69]. For both AfrLEA2 and AfrLEA3m, stripes of acidic residues were flanked by bands of basic residues along the length of the protein. In AfrLEA3m, the arrangement of charged residues formed parallel bands that extended linearly along the axis of the helix with a single sharp twist around residues 120–130 (Fig. 7A). To more clearly view the distinctive pattern made by charged amino acids in AfrLEA3m, the acidic and basic residues between amino acids 48–219 are shown with the side chains removed (Fig. 7B). Fig. 7C shows a front view of residues 149–240 with only the positive and negative residues displayed. Both panels B and C highlight the distinctive organization of acidic and basic amino acids found in AfrLEA3m, which are characteristic of class A α-helices originally described for apolipoproteins [48]. Somewhat differently compared to AfrLEA3m, where charged residues were arrange in a straight line for the majority of the protein's length, bands of charged amino acids in AfrLEA2 wrap around the axis of the helix for the entire length of the protein (Fig. 8A). As with AfrLEA3m, a smaller region of AfrLEA2 (amino acids 131–232) is displayed with the side chains removed (Fig. 8B) in order to emphasize the arrangement of charged residues. A front view of residues 29–98 also revealed an amphipathic motif similar to apolipoproteins (Fig. 8C).

4. Discussion

In this study we have shown that two LEA proteins from *A. franciscana* embryos, AfrLEA2 and AfrLEA3m, are effective at protecting liposomes with lipid compositions similar to biological membranes against damage incurred during desiccation. Liposomes with a non-biological composition of 100% phosphatidylcholine are not as effectively protected by either LEA protein, which is consistent with the mechanism of action for amphipathic proteins. The ability of AfrLEA3m or AfrLEA2 to stabilize liposomes does not appear to be dependent on lipid composition, provided there are physiological amounts of bilayer and non-bilayer-forming lipids present. Protection of IMM- and ILPM-like liposomes by AfrLEA3m and AfrLEA2 is significantly enhanced in the presence of the sugar trehalose, but this finding does not hold for OMM-like membranes. Thus additive protection by LEA proteins and trehalose is dependent on the lipid composition of the target membrane. In most cases, additional damage does not occur to liposomes dried for 7 days compared to overnight drying, which suggests that most damage occurs during the initial drying phase or during rehydration. Consistent with the ability to stabilize lipid bilayers, molecular modeling of the secondary structures for AfrLEA2 and AfrLEA3m reveals bands of charged amino acids similar to other amphipathic proteins previously documented to interact directly with membranes.

4.1. The effect of lipid composition on protection by LEA proteins

AfrLEA3m is predicted to be located in the matrix of *A. franciscana* mitochondria [40]. Therefore, the membrane with which AfrLEA3m

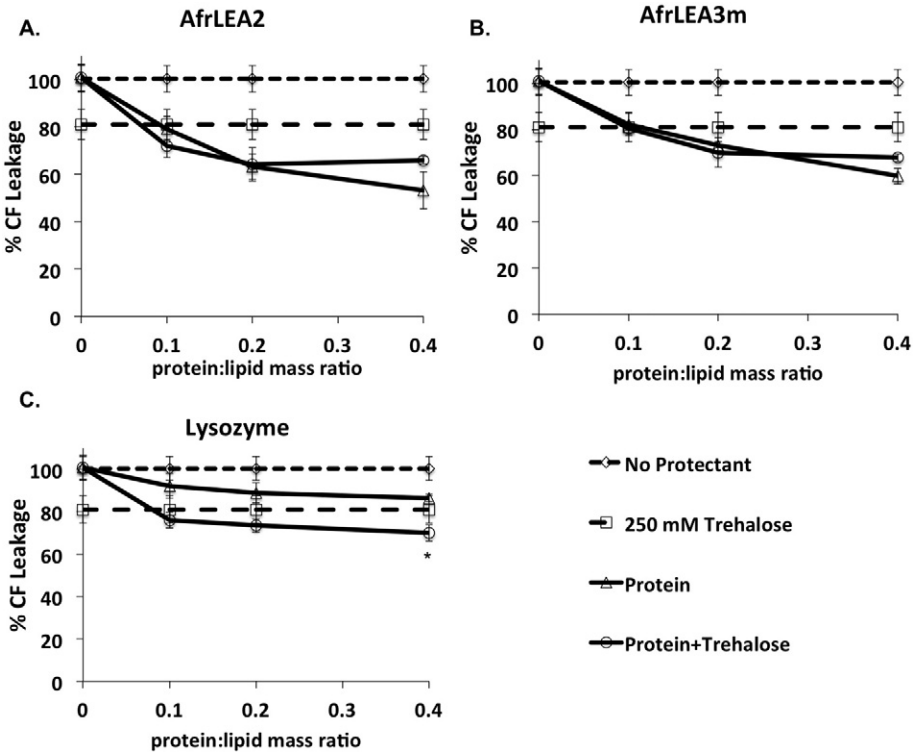

Fig. 4. Dye leakage from liposomes simulating the outer mitochondrial membrane dried overnight and rehydrated in the presence of trehalose, LEA proteins, and lysozyme. The relative stability of liposomes was determined by measuring the loss of entrapped carboxyfluorescein (CF). CF leakage was expressed as a percent of total CF loss, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were present at the indicated protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme for comparison to a non-stabilizing protein. Data represent mean ± SD of n = 6 samples; where error bars are absent, the SD was less than the size of the symbol. Asterisk indicates significantly greater protection by trehalose and protein combined than either component alone (2-way ANOVA, Bonferroni multiple comparisons test, $p \leq 0.05$, n = 6).

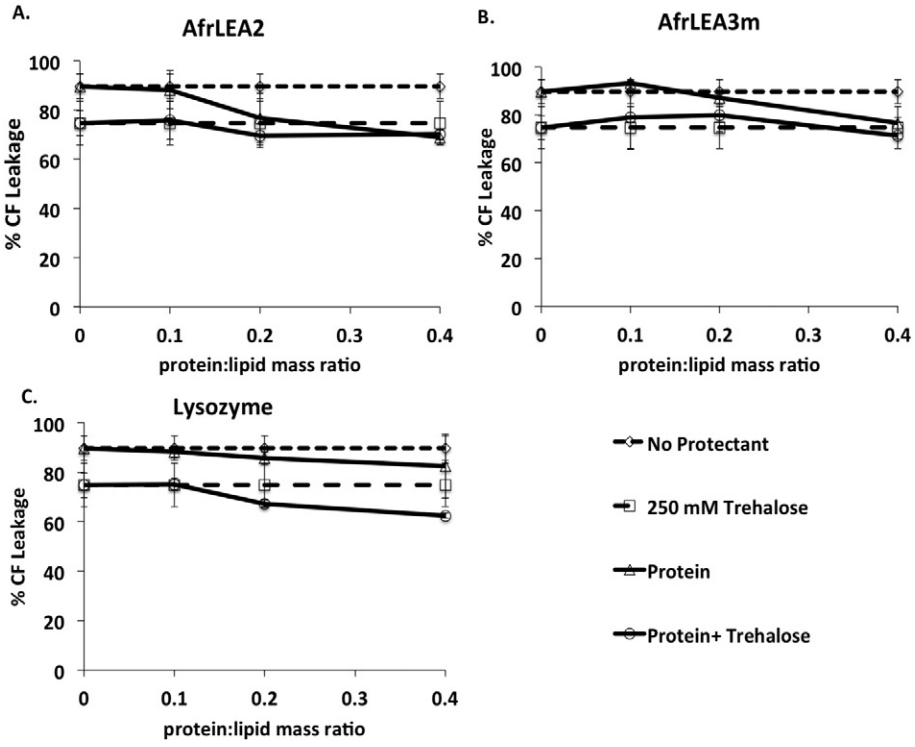

Fig. 5. Dye leakage from liposomes composed of 100% phosphatidylcholine dried overnight and rehydrated in the presence of trehalose, LEA proteins, and lysozyme. The relative stability of liposomes was determined by measuring the loss of entrapped carboxyfluorescein (CF). CF leakage was expressed as a percent of total CF loss, which was measured by detergent solubilization. (A) AfrLEA2 and (B) AfrLEA3m were added to drying media at physiologically relevant protein:lipid mass ratios in the presence and absence of 250 mM trehalose. (C) Liposomes were also dried with equal concentrations of lysozyme as a control protein. Data represent mean ± SD of n = 6 samples; where error bars are absent, the SD was less than the size of the symbol.

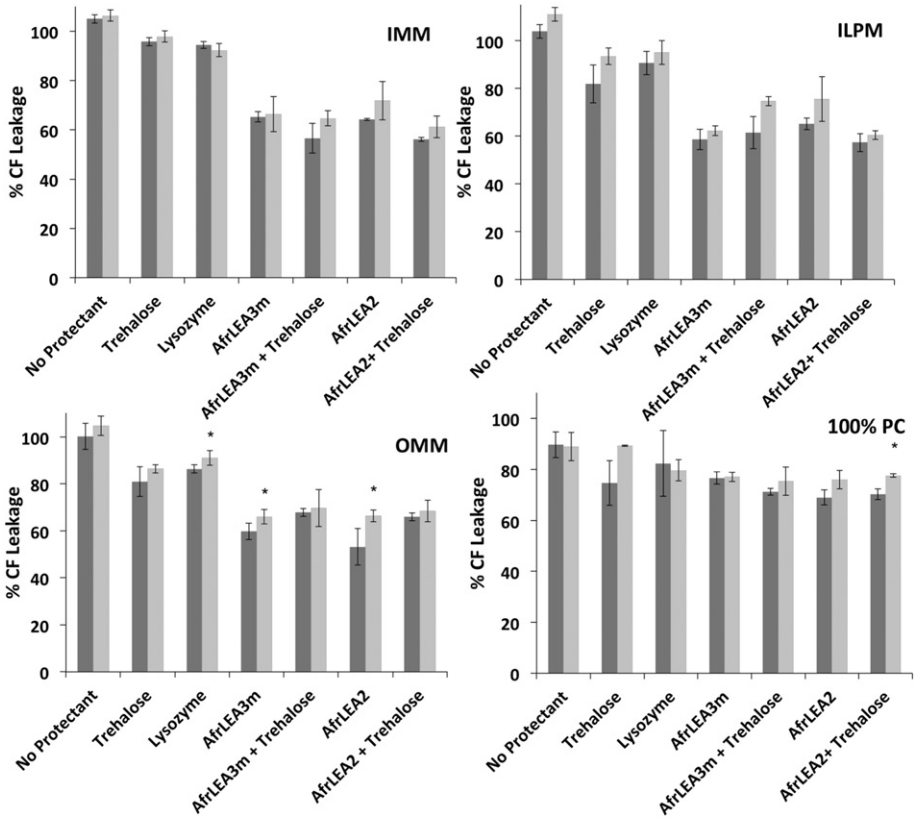

Fig. 6. Comparison of CF leakage from liposomes air-dried overnight (blue bars) or for 7 days (red bars) at room temperature with LEA proteins (0.4 protein:lipid mass ratio) and 250 mM external trehalose. Lipid compositions simulated that of the inner (IMM) and outer (OMM) mitochondrial membranes and the inner leaflet of the plasma membrane (ILPM), or a non-biological composition of 100% phosphatidylcholine (100% PC). Control values without additives (no protectant) are also presented. The relative stability of liposomes was assessed by measuring leakage of entrapped dye carboxyfluorescein (CF). CF leakage was expressed at a percentage of total CF loss, which was measured by detergent solubilization. Data represent mean ± SD of n = 3–6 samples. Asterisks indicate a significant difference between samples dried overnight or 7 days (Multiple t-tests, Holm–Sidak post hoc test, $p \leq 0.05$).

would be expected to primarily interact is the IMM. However, because AfrLEA3m is translated in the cytoplasm, it could potentially interact with the ILPM and OMM prior to import into mitochondria. To date, only models of the IMM and 100% PC membranes have been dried with a matrix-targeted LEA protein, specifically PsLEAm from the pea plant [32]. Logically, it was suggested by these investigators that the presence of cardiolipin, which is found predominantly in the IMM, might be important for facilitating interaction of the matrix-localized PsLEAm with this membrane. A similar specificity has been documented for a chloroplast-targeted stress protein (COR15) and its interactions with the chloroplast lipid monogalactosyldiacylglycerol for membrane stabilization during freezing [31]. Therefore, we hypothesized that AfrLEA3m, which is also matrix-targeted, would provide greater protection to IMM-like liposomes than to the OMM or ILPM liposomes. Instead, the data in our study indicate that AfrLEA3m provides similar protection to all three liposome types. Stabilization of ILPM liposomes is consistent with the observation that AfrLEA3m, when transfected into HepG2 cells, improves plasma membrane integrity during acute spin-drying and rehydration [47]. Liposome protection by AfrLEA2 was similar to that of AfrLEA3m for all types of liposomes tested, including the IMM. AfrLEA2, which is located in the cytoplasm [41], would not be expected to contact the IMM of intact mitochondria in vivo. Thus, it appears that AfrLEA2 and AfrLEA3m stabilize liposomes without bias for lipid composition, provided the liposomes mimic biological membranes. Note that our findings demonstrate LEA proteins are less effective at stabilizing 100% PC liposomes (non-biological), which was also the case with PsLEAm [32]; these results are similar to that reported for arbutin (a glycosylated hydroquinone), apparently due to the tight packing of PC in the hydrated state at high, non-physiological concentrations [76].

4.2. Additive protection of liposomes with combinations of trehalose and LEA proteins

The present study demonstrates for the first time the additive protection of lipid bilayers by trehalose plus LEA proteins. Protection of liposomes simulating the IMM and ILPM by the combination of 250 mM trehalose and AfrLEA2 or AfrLEA3m was greater than that afforded by either protectant alone. The lack of additional protection of OMM-like liposomes by combinations of trehalose and LEA protein indicates that cooperative stabilization by these components is dependent on the lipid composition of the lipid bilayer. At this point, the molecular explanation for this result is unclear. Nevertheless, previous results with stabilization of target enzymes during drying also show that additive protection by trehalose and LEA proteins is affected by the target enzyme chosen [28]. Additive protection of cellular components could partially explain why many, but not all [21,77,78], anhydrobiotic animals accumulate both trehalose and LEA proteins. Precisely how these protective molecules interact with each other is not known, although it has been shown that LEA proteins and model peptides of LEA proteins can stabilize sugar glasses [37,38]. The stability of sugar glasses corresponds to an increased glass transition temperature (T_g) as well as vitrification at higher water content [49]. Formation of sugar glasses is known to be important for preventing fusion, which results in passive leakiness of components entrapped within compartments formed by lipid bilayers [8,52].

4.3. Long term drying of liposomes with trehalose and LEA proteins

Under the majority of conditions tested herein, liposomes do not appear to undergo additional damage after seven days of dry storage at

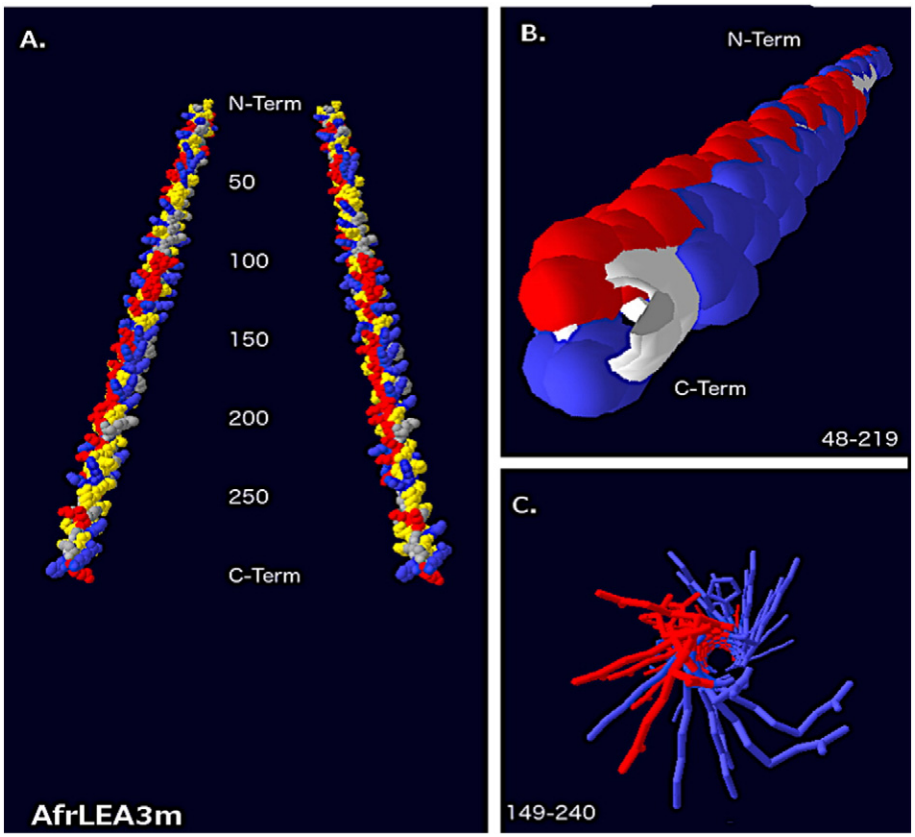

Fig. 7. Helical model of AfrLEA3m. (A) Two views of AfrLEA3m modeled as an α-helix with Chimera Software; images were generated with Swiss-PDB Viewer (DeepView, version 4.1). Charged amino acids are depicted in red (acidic: D or E) or blue (basic: H, K, or R). Hydrophobic (non-polar: A, G, I, L, M, V, or W) residues are colored gray and hydrophilic (polar: N, Q, S, T, or Y) residues are depicted as yellow. (B) The α-helical backbone (white) is depicted with the charged residues (colored as above) between positions 45–219. The three dimensional rendering of the side chains was removed to highlight the linear banding pattern formed by the residues. (C) End-on view of residues 149–240 with only the charged amino acids visible.

room temperature compared to liposomes dried overnight. A secondary implication from these findings is that water loss during desiccation was likely at steady-state after the overnight period of drying, since little further change in liposome stability was seen after 7 days. OMM-like liposomes were an exception; an increase in CF leakage occurred after extended drying in the presence of LEA proteins alone. While the addition of trehalose did not improve short-term protection of OMM liposomes dried with LEA proteins, it improved long-term stability of these liposomes in the dry state. The data indicate that during extended periods of desiccation, certain compositional types of membranes may be most effectively stabilized by a combination of trehalose and LEA protein, as opposed to either component alone.

4.4. Potential mechanisms for liposome stabilization by LEA proteins

Stabilization of IMM-like liposomes has been demonstrated with one other LEA protein, PsLEAm [32]. PsLEAm is a LEA protein found in the matrix of mitochondria from the pea plant, *P. sativum* [27]. There is evidence that PsLEAm stabilizes liposomes through direct interaction with the lipid bilayer [32]. Models of PsLEAm reveal regions where amino acids form parallel bands of charged residues [70]. These features allow PsLEAm to interact with membranes in a manner similar to that of amphipathic proteins [68,69]. The α-helix of amphipathic proteins aligns perpendicular to the phospholipid molecules (i.e., lies flat against the membrane surface), such that the protein settles in between the phospholipid head groups. The positively charged amino acids interact with the negatively charged phospholipid headgroups, and the nonpolar residues face the hydrophobic core of the membrane [68,69]. This positioning of LEA proteins between polar headgroups is thought to maintain spacing between lipids and increase acyl-chain mobility at low water contents, which results in depression of the T_m [32]. Furthermore, non-bilayer forming lipids such as PE and CL promote inverted hexagonal II (Hex_{II}) phase transitions in lamellar membranes, a problem exacerbated by low water content [71,72]. Insertion of amphipathic molecules between phospholipid headgroups is thought to alleviate membrane inversion stress and promote retention of the lamellar phase [6,34,35]. Indeed freezing studies show that COR15 proteins (LEA23 and LEA24) are able to retard the transition from lamellar to Hex_{II} phase in chloroplasts [34,35]. Interestingly, protective sugars are less effective at stabilizing membranes that contain certain non-bilayer forming lipids, and in some cases the sugars exacerbate water stress-induced damage [73–75]. The ability to stabilize membranes with a high representation of non-bilayer forming lipids may be one advantage for the accumulation of LEA proteins in cells despite the presence of protective sugars. In future experiments it could be revealing to dry liposomes with LEA protein present on both sides of the lipid bilayer, which to our knowledge has not been attempted thus far.

At present biophysical evidence supporting direct interaction of AfrLEA2 and AfrLEA3m with phospholipid bilayers is lacking. Nevertheless molecular modeling of these two proteins (Figs. 7, 8) reveals regions where amino acids of positive and negative charge align in parallel bands, with acidic (negative) residues flanked to either side by basic (positive) residues. The arrangement of charged amino acids closely resembles that of PsLEAm [70]. Such an organization of charged amino acids allows PsLEAm to directly interact with lipid bilayers, presumably in a manner similar to other amphipathic proteins [16,70]. Considering their shared structural features are so distinctive, we hypothesize that AfrLEA2 and AfrLEA3m may interact with lipid bilayers in a manner similar to PsLEAm and other amphipathic helices. It is appropriate to note that formation of bands of charged amino acids does

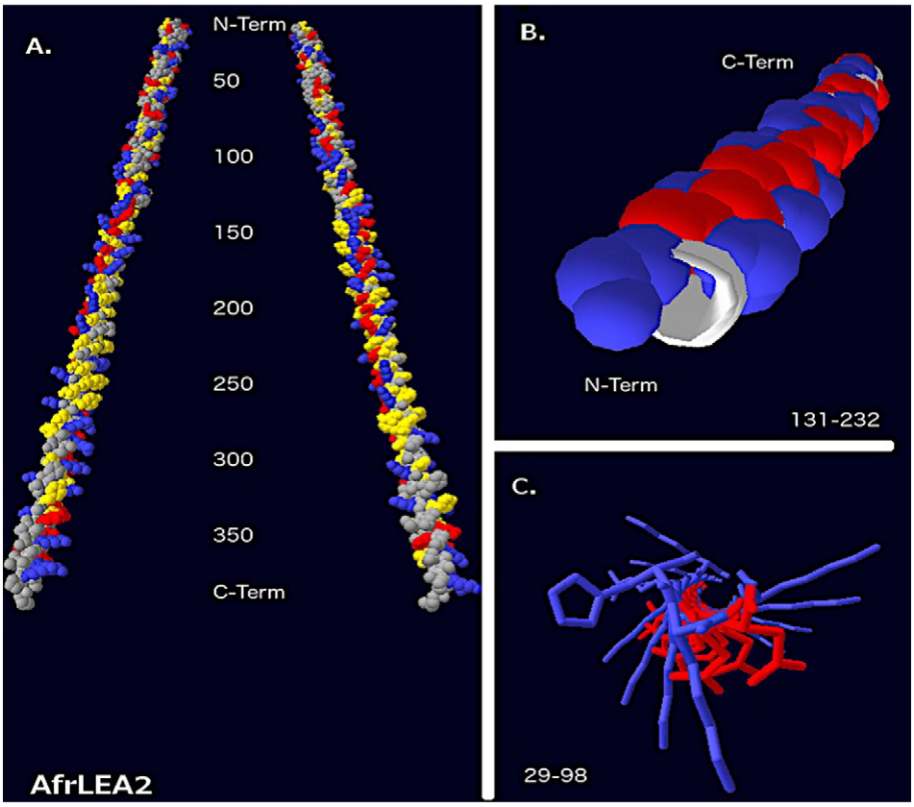

Fig. 8. Helical model of AfrLEA2. (A) Two views of AfrLEA2 modeled as an single helical rod. The amino acid sequence for AfrLEA2 was modeled as an alpha helix with Chimera Software and images were generated with Swiss-PDB Viewer (DeepView, version 4.1). Charged amino acids are depicted in red (acidic: D or E) or blue (basic: H,K, or R). Hydrophobic (non-polar: A,G, I, L, M, V, or W) residues are colored gray and hydrophilic (polar: N, Q, S, T, or Y) residues are depicted as yellow. (B) The α-helical backbone (white) is depicted with the charged residues (colored as above) between positions131-232. The three dimensional rendering of the side chains was removed to highlight the linear banding pattern formed by the residues. (C) End-on view of residues 29–98 with only the charged amino acids visible.

not necessarily mean that a LEA protein will stabilize lipid bilayers. Another LEA protein with similar structural features, LEA7 from *A. thaliana*, interacts with lipid bilayers but does not improve the stability of air-dried liposomes [33,79]. Therefore, while molecular models of LEA proteins are valuable for predicting the function of the protein, it is necessary to experimentally demonstrate liposome stabilization to confirm that a specific LEA protein indeed protects lipid bilayers, as we have done here, and further to test for direct interaction with phospholipid bilayers as we will evaluate in subsequent studies.

5. Conclusions

LEA proteins and trehalose are accumulated in the desiccation-tolerant embryos of *A. franciscana*. In this study we demonstrate that two LEA proteins from *A. franciscana*, AfrLEA2 and AfrLEA3m, stabilize liposomes that mimicked biological membranes when air dried overnight and for one week. Neither the cytoplasmic-localized AfrLEA2 nor the mitochondrial-targeted AfrLEA3m exhibits preferential protection for one compositional type of liposome over another (outer mitochondrial membrane, inner mitochondrial membrane, inner leaflet of the plasma membrane). For example, matrix-resident AfrLEA3m is not more proficient at stabilizing IMM-like liposomes that contain cardiolipin than is AfrLEA2. When trehalose and LEA proteins are used in combination, IMM-like liposomes and ILPM liposomes are protected to a greater degree than when dried with either protectant alone. Modeling of AfrLEA2 and AfrLEA3m as α-helices reveals arrangements of charged amino acids that are consistent with other amphipathic proteins previously shown to directly interact with lipid bilayers. Our study reveals cooperative stabilization of lipid bilayers by multiple types of protectants as has been observed for protein stabilization [28,30].

Acknowledgments

This research was supported by the National Science Foundation grants IOS-0920254 and IOS-1457061/IOS-1456809. We would like to thank Prof. Ramachandra Devireddy, Department of Mechanical and Industrial Engineering, for help regarding liposome preparation.

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